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EUROPEAN PATENT SPECIFICATION

- 45 Date of publication of patent specification: **04.09.91** 51 Int. Cl.⁵: **C12Q 1/68, G01N 33/533**
- 21 Application number: **86116652.8**
- 22 Date of filing: **01.12.86**

54 **Fluorescent Stokes shift probes for polynucleotide hybridization assays.**

- 30 Priority: **23.12.85 US 812111**
- 43 Date of publication of application:
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- 45 Publication of the grant of the patent:
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- 56 References cited:
EP-A- 0 070 685
EP-A- 0 144 913
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PATENT ABSTRACTS OF JAPAN, vol. 10, no. 149 (P-461)[2206], 30th May 1986; & JP - A - 59 123 757 (FUJI REBIO K.K.) 09-01-186

PROCEEDINGS NATIONAL ACADEMY OF SCIENCES USA, vol. 58, 1967, pages 719-729, Washington, US; L. STRYER et al.: "Energy transfer: a spectroscopic ruler"

CLINICAL CHEMISTRY, vol. 29, no. 9, September 1983, pages 1582-1586, Washington, US; M.N. KRONICK et al.: "Immunoassay tech-

niques with fluorescent phycobiliprotein conjugates"

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dependence of the transfer efficiency on distance was in excellent agreement with the dependence predicted by the Förster equations. The results were so close to theoretical predictions that the authors proposed use of non-radiative energy transfer as a spectroscopic ruler. Related experiments with model systems reported by other researchers are confirmatory. See, for example, Gabor, Biopolymers 6:809-816 (1968); and Katchalski-Katzir, et al., Ann. N. Y. Acad. Sci. 366: 44-61 (1981). The use of the Förster energy transfer effect has been described in the following immunofluorescent assay patents. (See U. S. Patent Nos. 3,996,345; 3,998,943; 4,160,016; 4,174,384; and 4,199,599). The energy transfer immunofluorescent assays described in these patents are based on the decrease or quenching of the donor fluorescence rather than fluorescent re-emission by the acceptor [Ullman, E. F., et al., J. Biol. Chem., Vol. 251, 14, pp. 4172-4178 (1976)].

Homogeneous immunoassay procedures based on chemiluminescent labels or bioluminescent proteins have been reported which involve non-radiative energy transfer, see Patel, et al., Clin. Chem. 29 (9):1604-1608 (1983); and European Patent Application 0 137 515, published April 17, 1985. By close spacing of the donor-acceptor group according to the principles of non-radiative energy transfer for high transfer efficiency it was proposed that homogeneous assays could be made practical. Homogeneous assays are inherently simpler to carry out but their use had been subject to the limitation that unbound labelled probe remains in solution and causes interfering background signal. European Patent Application 0 137 515 published April 17, 1985 refers to various ligand-ligand interactions which can be used with the bioluminescent proteins including nucleic acid-nucleic acid interactions. The examples, however, are directed to protein ligands rather than nucleic acids.

European Patent Application 0 070 685, published January 26, 1983, relates to homogeneous nucleic acid hybridization assays employing non-radiative energy transfer between absorber/emitter moieties positioned within 10 nm (100 Angstroms) of each other. As described, the hybridization probes are prepared by attaching the absorber-emitter moieties to the 3' and 5' end units of pairs of single-stranded polynucleotide fragments derived from DNA or RNA by restriction enzyme fragmentation. The pairs of polynucleotide fragments are selected to hybridize to adjacent complementary sequences of the target polynucleotide with the labelled ends with no overlap and with few or no base-pairing spaces left between them. The preferred donor moiety is a chemiluminescent catalyst and the absorber moiety is a fluorophore or phosphore.

THE DRAWINGS

FIGURES 1 to 5 illustrate preferred embodiments of Stokes shift probes for use in practicing the invention.

SUMMARY OF INVENTION

This invention is based in part on the discovery that polynucleotides (DNA or RNA) provide an environment which strongly influences non-radiative energy transfer between donor-acceptor fluorescent moieties attached to polynucleotide probes. Prior to the present invention, it was not known how to design fluorophore-labelled probes with donor-acceptor moieties for practical and effective use with polynucleotides, particularly with regard to efficient emission by the acceptor fluorophore. It has been found that a novel spacing of the fluorescent moieties is critical for maximizing energy transfer and producing highly efficient fluorescent emission by the acceptor. Surprisingly, the optimum spacing requires intervening base pair units between the nucleotides to which the fluorescent moieties are attached. In particular, contrary to prior knowledge about Förster non-radiative energy transfer attachment of the fluorescent moieties to immediately adjacent nucleotide units (donor/acceptor distance 1.0-1.5 nm (10 - 15 Å)) or with only a single intervening unit results in an unacceptably low transfer efficiency. The theoretical explanation for this new phenomenon is not known. However, it apparently relates to the formation of excitation traps when the fluorescent probe(s) is hybridized to the target polynucleotide. This "microenvironment" of the helical double-stranded polynucleotides has a marked effect on the optimum spacing for non-radiative energy transfer and efficient fluorescent emission by the acceptor.

More specifically, it has been found that for efficient acceptor emission the donor-acceptor fluorescent moieties should be separated when hybridized by at least two intervening base units but not over seven units. For optimum efficiency with either single probe or dual probe embodiments a separation range of from 3 to 6 base units is preferred. To maximize the benefits of this invention, the linker arm side chains which connect the fluorescent moieties to the nucleic acid (pyrimidine or purine) base units should have lengths within the range from 0.4 to 3.0 nm (4 to 30 Angströms (Å)) and preferably from about 1.0 to 2.5

dependence of the transfer efficiency on distance was in excellent agreement with the dependence predicted by the Förster equations. The results were so close to theoretical predictions that the authors proposed use of non-radiative energy transfer as a spectroscopic ruler. Related experiments with model systems reported by other researchers are confirmatory. See, for example, Gabor, *Biopolymers* 6:809-816 (1968); and Katchalski-Katzir, et al., *Ann. N. Y. Acad. Sci.* 366: 44-61 (1981). The use of the Förster energy transfer effect has been described in the following immunofluorescent assay patents. (See U. S. Patent Nos. 3,996,345; 3,998,943; 4,160,016; 4,174,384; and 4,199,599). The energy transfer immunofluorescent assays described in these patents are based on the decrease or quenching of the donor fluorescence rather than fluorescent re-emission by the acceptor [Ullman, E. F., et al., *J. Biol. Chem.*, Vol. 251, 14, pp. 4172-4178 (1976)].

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of the visible spectrum (>600nm).

Fluorescein is a particularly desirable donor moiety. Lucifer Yellow can also be employed as a donor moiety, particularly in combination with Texas Red as an acceptor moiety. The emission spectra of fluorescein (EX~492nm, EM~520nm, EC~70,000, QY high) and of Lucifer Yellow (EX~428nm, EM~540nm, EC~12,000, QY medium) both sufficiently overlap the excitation spectrum of Texas Red (EX~590nm, EM~615nm, EC~70,000, QY high). Fluorescein's excitation maximum (~492nm) comes very close to the 488nm Argon laser line and Lucifer Yellow's excitation maximum (~428nm) comes very close to the 442nm Helium-Cadmium laser line. In addition the fluorescein/Texas Red and Lucifer Yellow/Texas Red combinations provide large Stokes shifts of ~130nm and ~170nm respectively. In both cases the 615nm to 620nm Texas Red emission is at significantly higher wavelengths than the Raman water lines (~585 nm for 448nm excitation and ~520nm for 442nm excitation). As compared with the use of a fluorescein reporter group alone, the combination with a Texas Red acceptor provides a ten to twenty fold increase in the relative detection sensitivity in the 615nm to 620nm emission region for excitation at ~490nm. As compared with the use of Lucifer Yellow group alone, the combination with a Texas Red acceptor provides two to three fold increase in relative detection sensitivity in the 615nm to 620nm emission region.

Fluorescein fluorophores can be incorporated in the polynucleotide probe as a fluorescein isothiocyanate derivative obtainable from Molecular Probes, Inc., Junction City, Oregon, or Sigma Chemical Co., St. Louis, Missouri. Texas Red sulfonyl chloride derivative of sulforhodamine 101 is obtainable from Molecular Probes, Inc. Texas Red can also be prepared from sulforhodamine 101 by reaction with phosphorous oxychloride, as described in Titus, et al., *J. Immunol. Meth.*, 50, pp. 193-204, 1982. Lucifer Yellow is obtainable from Aldrich Chemical Co., Milwaukee, Wisconsin, as the vinyl sulfone derivative (Lucifer Yellow VS). Lucifer Yellow VS is a 4-amino-N-[3-vinylsulfonyl] phenyl]naphthalimide-3, 5-disulfonate fluorescent dye. For a description of its use, see Stewart, W., *Nature*, Vol. 292, pp. 17-21 (1981).

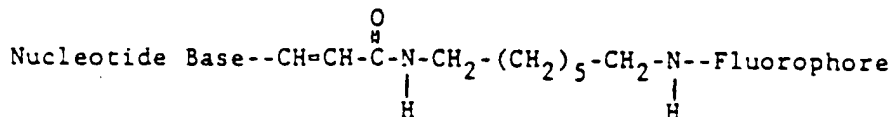
The foregoing description should not be understood as limiting the present invention to combinations of fluorescein with Texas Red or Lucifer Yellow with Texas Red. Those combinations preferred by the principles of the invention are more broadly applicable. The spacing feature of this invention can be utilized with other donor-acceptor pairs of fluorophores. For example, with fluorescein and Lucifer Yellow as donors, the acceptor fluorophore moieties prepared from the following fluorescent reagents are acceptable: Lissamine rhodamine B sulfonyl chloride; tetramethyl rhodamine isothiocyanate; rhodamine x isothiocyanate; and erythrosin isothiocyanate. Other suitable donors to the acceptors listed above (including Texas Red) are B-phycoerythrin and 9-acridineisothiocyanate derivatives.

When fluorescein is used as the acceptor moiety then suitable donors can be obtained from Lucifer Yellow VS; 9-acridine-isothiocyanate; 4-acetamido-4'-isothio-cyanatostilbene-2,2'-disulfonic acid; 7-diethylamino-3-(4'-isothiocyanatophenyl)-4-methylcoumarin.

When diethylenetriamine pentaacetate or other chelates of Lanthanide ions (Europium and Terbium) are used as acceptors, then suitable donors can be obtained from succinimidy 1-pyrene-butyrate; and 4-acetamido-4'-isothiocyanatostilbene-2,2'-disulfonic acid derivatives.

Linker Arms

The length of the linker arms connecting the fluorescent moieties to the base units of the probes is also an important parameter for obtaining the full benefit of the present invention. The length of the linker arms for the purpose of the present invention is defined as the distance in Angstroms from the purine or pyrimidine base to which the inner end is connected to the fluorophore at its outer end. In general, the arm should have lengths of not less than 0.4 nm (4) nor more than 3.0 nm (30Å). The preferred length of the linker arms is from 1.0 to 2.5 nm (10 to 25Å). The linker arms may be of the kind described in PCT application WO 84.03285. That application discloses a method for attaching the linker arms to the selected purine or pyrimidine base and also for attaching the fluorophore to the linker arm. The linker arm represented below is illustrative of the linker arms which may be employed for the purposes of the present invention as further described in the cited PCT application.



The linker arm as represented above contains 12 units in the chain and has a length of approximately

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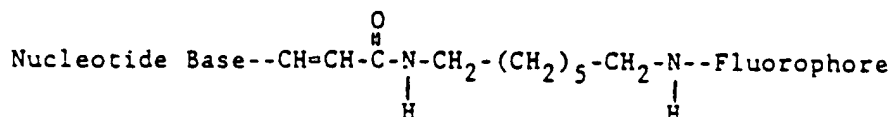
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The linker arm as represented above contains 12 units in the chain and has a length of approximately

Example I

By way of specific illustration, the preparation of a polynucleotide probe containing a fluorescein and a Texas Red moiety with an $n = 5$ spacing can be carried out as follows. The starting material is approximately 300 μ g of the appropriate synthetic (25mer) polynucleotide probe containing two primary amine functionalized linker arm nucleotides separated by five nucleotides within the sequence. The 300 μ g of polynucleotide is taken up in about 20 μ l of 0.5M sodium bicarbonate buffer at pH 8.8. About 100 μ g of Texas Red dissolved in 10 μ l of water is added to the polynucleotide solution. A limited reaction is carried out at 0-5 $^{\circ}$ C for approximately 15 minutes. At this point about 10 μ l of a 7M urea solution is added and the reaction mixture is separated over a 0.7cm x 3.0cm G-25 Sephadex Column. The initial fractions (excluded volume) contain the unreacted polynucleotide, mono-substituted Texas Red polynucleotide probe, and di-substituted Texas Red polynucleotide probe. The final fractions (included volume) contain the unreacted Texas Red. The initial fractions are pooled and lyophilized, and the final fractions are discarded. The lyophilized pooled fractions are brought up in a small volume (5-10 μ l) of 3.5M urea for separation by gel electrophoresis.

Electrophoresis on a 20% polyacrylamide gel (7-8M urea) separates the sample into three distinct bands, the lower is the unreacted polynucleotide, the middle band is the mono-substituted Texas Red polynucleotide, and the upper band is the di-substituted Texas Red polynucleotide. Reaction conditions were originally controlled in order to prevent total conversion of the polynucleotide to the di-substituted Texas Red polynucleotide derivative. At this point the band containing the mono-substituted Texas Red polynucleotide derivative is carefully excised from the gel and the derivative is extracted with water, and the resulting solution lyophilized to dryness. The lyophilized sample is now taken up in a small volume of water and desalted on a G-25 Sephadex column. The fractions containing the mono-substituted Texas Red polynucleotide probe are pooled and lyophilized.

The sample is now ready for the second reaction to incorporate the fluorescein moiety into mono-substituted Texas Red polynucleotide probe. The sample is again taken up in about 20 μ l of 0.5M sodium bicarbonate buffer at pH 8.8. About 500 μ g of fluorescein isothiocyanate (FITC) in 10 μ l water is added to the buffered solution containing the mono-substituted Texas Red polynucleotide probe. The reaction is carried out at 0-5 $^{\circ}$ C for about two hours. About 10 μ l of a 7M urea solution is added, and the sample is run over another G-25 Sephadex column, as described previously, to separate reacted polynucleotide probe from FITC. Again appropriate fractions are pooled and lyophilized. The sample is again electrophoresed on a 20% polyacrylamide gel, separating the sample into two bands; the lower being unreacted mono-substituted Texas Red polynucleotide probe and the upper band being the fluorescein and Texas Red substituted polynucleotide probe. The upper band is carefully excised, extracted, lyophilized, and desalted on a G-25 Sephadex column as was described above. The final purified fluorescein-Texas Red polynucleotide probe is then analyzed by UV Visible spectroscopy. The ratio of adsorption (O.D.) at 260nm, 492nm, and 592nm can be used to determine proper stoichiometry for the probe; the 25mer polynucleotide probe contains one fluorescein and one Texas Red moiety.

The synthesis and purification of probes containing a single fluorophore is straightforward. The starting material is a 25mer polynucleotide probe containing only one amine functionalized linker arm nucleotide incorporated at the appropriate position within the probe. In the case of both Texas Red and FITC, the reactions are carried out for a longer time (about two hours) in order to increase yield of the fluorophore substituted probe. Subsequent steps for purification are the same as those described above.

Example II

A series of fluorescein-Texas Red 25mer polynucleotide probes (F&TR probes) were prepared in which the separation between the fluorophore moieties was $n=0$, $n=1$, $n=5$, $n=6$, $n=9$, and $n=12$. The probes were designed to hybridize to Herpes Simplex Virus (type 1) target DNA. The procedure was as described in Example I using the 1.4 nm (14 \AA) linker arm previously illustrated. The actual sequence and relative position of fluorophores in the $n=5$, F&TR probe is shown below.



It should be pointed out that the fluorophores can occupy either linker arm position on the probe. But

Example I

By way of specific illustration, the preparation of a polynucleotide probe containing a fluorescein and a Texas Red moiety with an $n = 5$ spacing can be carried out as follows. The starting material is approximately 300 μ g of the appropriate synthetic (25mer) polynucleotide probe containing two primary amine functionalized linker arm nucleotides separated by five nucleotides within the sequence. The 300 μ g of polynucleotide is taken up in about 20 μ l of 0.5M sodium bicarbonate buffer at pH 8.8. About 100 μ g of Texas Red dissolved in 10 μ l of water is added to the polynucleotide solution. A limited reaction is carried out at 0-5 $^{\circ}$ C for approximately 15 minutes. At this point about 10 μ l of a 7M urea solution is added and the reaction mixture is separated over a 0.7cm x 3.0cm G-25 Sephadex Column. The initial fractions (excluded volume) contain the unreacted polynucleotide, mono-substituted Texas Red polynucleotide probe, and di-substituted Texas Red polynucleotide probe. The final fractions (included volume) contain the unreacted Texas Red. The initial fractions are pooled and lyophilized, and the final fractions are discarded. The lyophilized pooled fractions are brought up in a small volume (5-10 μ l) of 3.5M urea for separation by gel electrophoresis.

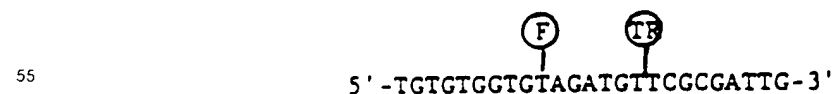
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Table A also shows that the results for the unhybridized F&TR probe series are similar to the hybridized series, but less pronounced. Again, the $n=0$ and $n=1$ values are lower than would be expected from the Förster equation. The highest observed efficiency value is 50, and this is for the $n=9$ F&TR probe. It appears that hybridization provides an improved environment which leads to a higher overall observed energy transfer efficiency at or near the $n=5$ position, as well as causing a lowering of efficiency at the $n=0$ and $n=1$ positions.

Example III

Two sets of dual 25mer probes were prepared using the same procedure and linker arms as in Example III. The final separation of fluorescein and Texas Red (upon hybridization to target polynucleotide) was $n=0$ and $n=6$ base pair units.

Results for the fluorescent analysis of Probe Set 1 ($n=0$) and Probe Set 2 ($n=6$) hybridized to complementary target polynucleotide are given in Table B:

TABLE B

Observed Energy Transfer Efficiencies
for Dual Probes (Fluorescein Probe and
Texas Red Probe) Hybridized to Target Polynucleotide

Probe Set	Base Pair Separation (n)	(Emission 615nm, Excitation 490nm)
1	0	15
2	6	52

The results in Table B show that the observed energy transfer efficiency is highest for Probe Set 2 ($n=6$) and unexpectedly lower for Probe Set 1 ($n=0$). The results for the dual probe system corroborate the results obtained for the single F&TR probe series. Again, the dual probe results indicate that there is a unique narrow range of optimal positions around the $n=6$ base pair spacing.

Example IV

A 25mer probe containing Lucifer Yellow as the donor and Texas Red as the acceptor (LY&TR probe) with an $n=5$ nucleotide spacing was prepared using the basic procedures described earlier. Observed energy transfer efficiency, in terms of emission at 615nm when excited at 435nm, was found to be approximately 20%. The relative value is lower than the value of 82% for the $n=5$ F&TR probe. The lower "relative" value is due to the fact that the extinction coefficient of Lucifer Yellow is significantly lower than fluorescein, ~12,000 for Lucifer Yellow versus 75,000 for fluorescein. Lucifer Yellow because of this property is not as "good" a donor as fluorescein. However, the Lucifer Yellow/Texas Red pair produces a large Stokes shift (~170nm) and the donor can be excited by a laser (Helium-Cadmium, ~442nm).

Example V

The example detailed here concerns the use of single probes of Example II used in a sandwich type heterogeneous assay format to detect Herpes Simplex Virus DNA. By way of background the sandwich type assay involves the initial capture, via hybridization, of the given target polynucleotide by a complementary probe (capture probe) immobilized on some type of support material (polystyrene or Agarose beads). The now captured (immobilized by hybridization) target polynucleotide is contacted with another complementary probe which has been labelled with a reporter group (fluorophore, etc.). The now hybridized reporter probe signals the presence of the target polynucleotide sequence.

In the assay procedure about 50 to 100 Agarose Herpes Simplex Virus (HSV) capture beads (~100 micron diameter) are employed. The Agarose HSV capture beads were prepared by substitution (covalent linkage) of appropriate complementary HSV probes (20-50 nucleotides in chain length) to an activated form

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in length, and said linker arms have lengths of 1,0 to 2,5 nm (10 to 25 Angstroms).

7. The method of claim 6 in which said donor and acceptor moieties are on a single probe.
- 5 8. The method of claim 6 in which said donor and acceptor moieties are on a pair of probes.
9. The method of claims 6, 7, or 8 in which said donor moiety is fluorescein and said acceptor moiety is Texas Red.
- 10 10. The method of claims 1 or 6 in which said donor and acceptor moieties are on a single probe and are connected to base units thereof other than the 3' and 5' end units and which are separated by 4 to 6 intervening base units.
- 15 11. The method of claims 1 or 6 in which said moieties are on a pair of probes respectively having 3' and 5' ends hybridizing to adjacent base units of the target polynucleotide and when so hybridized the donor and acceptor moieties being separated by 4 to 6 intervening base units.
12. The methods of claims 10 or 11 in which said donor moiety is fluorescein and said acceptor moiety is Texas Red.

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Revendications

1. Procédé spectroscopique de détection d'une séquence polynucléotidique monocaténaire - cible dans un échantillon de polynucléotides, selon lequel (i) une seule séquence-sonde polynucléotidique fluorescente complémentaire de ladite séquence-cible est hybridée avec cette dernière, ou (ii) plusieurs sondes formées par des séquences complémentaires de parties voisines successives de ces séquences-cibles sont hybridées avec ces dernières, caractérisé en ce qu'au moins une paire de groupes fluorescents liés par l'intermédiaire de segments de liaison à des motifs basiques dérivés d'acide nucléique, sont présents dans ladite sonde unique ou dans ladite pluralité de sondes, ces groupes fluorescents comprenant respectivement des groupes donneur et accepteur sélectionnés de façon à ce que le spectre d'émission du groupe donneur chevauche le spectre d'excitation du groupe accepteur, afin de permettre un transfert d'énergie non radiatif avec une émission efficace de fluorescence de la part du fluorophore accepteur, la longueur d'onde maximum du spectre d'émission du groupe accepteur, étant d'au moins 100 nm supérieure à la longueur d'onde maximum du spectre d'excitation du groupe donneur, les segments de liaison ayant des longueurs allant de 0,4 à 3,0 nm (de 4 à 30 Å), les groupes donneur et accepteur étant liés à des motifs basiques non contigus dans ladite sonde unique, ou dans ladite pluralité de sondes à des motifs basiques autres que les motifs des extrémités 3' et 5' de celles-ci, et en ce que lorsque l'unique sonde ou ladite pluralité de sondes sont hybridées à l'échantillon-cible, les motifs basiques auxquels les groupes donneur et accepteur sont liés, sont appariés par hybridation avec les motifs basiques de ladite séquence-cible qui sont séparés par 2 à 7 motifs basiques nucléotidiques intercalaires de la séquence-cible.
2. Procédé selon la revendication 1, dans lequel les groupes donneur et accepteur sont incorporés dans une seule sonde.
3. Procédé selon la revendication 1, dans lequel les groupes donneur et accepteur sont incorporés dans une paire de sondes.
4. Procédé selon la revendication 1, 2 ou 3, dans lequel le groupe donneur est la fluorescéine, et le groupe accepteur est le rouge Texas.
5. Procédé selon la revendication 1, dans lequel, lorsque lesdites sondes uniques ou ladite sonde double sont hybridées avec la séquence-cible, les motifs basiques liés aux groupes donneur et accepteur, sont appariés avec des motifs basiques nucléotidiques de la séquence-cible, qui sont séparés par 3 à 6 motifs basiques intercalaires.
6. Procédé selon la revendication 1, dans lequel les sondes sont des polynucléotides synthétiques d'une longueur de 10 à 100 motifs basiques, et dans lequel les segments de liaison ont des longueurs de 1,0

à 2,5 nm (de 10 à 25 Å).

7. Procédé selon la revendication 6, dans lequel les groupes donneur et accepteur sont incorporés dans une sonde unique.
8. Procédé selon la revendication 6, dans lequel les groupes donneur et accepteur sont incorporés dans une paire de sondes.
9. Procédé selon la revendication 6, 7 ou 8, dans lequel le groupe donneur est la fluorescéine, et le groupe accepteur est le rouge Texas.
10. Procédé selon la revendication 1 ou 6, dans lequel les groupes donneur et accepteur sont incorporés dans une unique sonde et sont liés à des motifs basiques de celle-ci, autres que les motifs des extrémités 3' et 5', et séparés par 4 à 6 motifs basiques intercalaires.
11. Procédé selon la revendication 1 ou 6, dans lequel les groupes sont incorporés dans une paire de sondes dont les extrémités 3' et 5' sont respectivement hybridées à des motifs basiques adjacents du polynucléotide-cible, et les groupes donneur et accepteur hybridés de cette façon étant séparés par 4 à 6 motifs basiques intercalaires.
12. Procédé selon la revendication 10 ou 11, dans lequel le groupe donneur est la fluorescéine, et le groupe accepteur est le rouge Texas.

Patentansprüche

1. Spektroskopisches Verfahren zum Nachweis einer einzelsträngigen Zielpolynukleotidsequenz in einer Polynukleotidprobe, bei dem entweder (i) eine fluoreszierende Polynukleotideinzelsonde, deren Sequenz komplementär zu der Zielsequenz ist, mit dieser hybridisiert, oder (ii) eine Vielzahl von Sonden solcher Sequenzen, die komplementär zu in der Sequenz benachbarten Anteilen der Zielsequenzen sind, mit diesen hybridisieren, **dadurch gekennzeichnet**, daß in der Einzelsonde oder in der Vielzahl von Sonden mindestens ein Paar fluoreszierender Gruppen vorhanden ist, die durch Verbindungsarme an Nukleinsäurebaseneinheiten gebunden sind, wobei die fluoreszierenden Gruppen Donor- bzw. Akzeptorgruppen umfassen, die so ausgewählt sind, daß das Emissionsspektrum der Donorgruppe das Anregungsspektrum der Akzeptorgruppe überlappt, um einen strahlungsfreien Energietransfer mit einer effizienten Fluoreszenzemission vom Akzeptor-Fluorophor zu erlauben, wobei das Wellenlängenmaximum des Emissionsspektrums der Akzeptorgruppe mindestens 100 nm größer als das Wellenlängenmaximum des Anregungsspektrums der Donorgruppe ist und wobei die Verbindungsarme Längen von 0,4 - 3,0 nm (4 - 30 Angström) haben und die Donor- und Akzeptorgruppen in der Einzelsonde an nicht benachbarte Baseneinheiten oder in der Vielfachsonde an andere Baseneinheiten als deren 3'- und 5'-Ende gebunden sind, wobei, wenn die Einzelsonde oder die Vielfachsonden mit der Zielprobe hybridisiert werden, die Baseneinheiten, an die die Donor- und Akzeptorgruppen gebunden werden, durch Hybridisierung mit Baseneinheiten der Zielsequenz verbunden werden, die durch 2 bis 7 dazwischenliegende Nukleotidbaseneinheiten der Zielsequenz getrennt sind.
2. Verfahren nach Anspruch 1, in dem die Donor- und Akzeptorgruppen auf einer Einzelsonde sind.
3. Verfahren nach Anspruch 1, in dem die Donor- und Akzeptorgruppen auf einem Paar von Sonden sind.
4. Verfahren nach Anspruch 1, 2 oder 3, in dem die Donorgruppe Fluorescein und die Rezeptorgruppe Texasrot ist.
5. Verfahren nach Anspruch 1, in dem, wenn die Einzelsonde oder die Doppelsonde mit der Zielsequenz hybridisiert ist, die Baseneinheiten, die mit den Donor- und Akzeptorgruppen verbunden sind, mit Nukleotidbaseneinheiten der Zielsequenz gepaart sind, die durch 3 bis 6 dazwischenliegende Baseneinheiten getrennt sind.
6. Verfahren nach Anspruch 1, in dem die Sonden synthetische Polynukleotide mit 10 - 100 Baseneinheiten

ten Länge sind und die Verbindungsarme Längen von 1,0 - 2,5 nm (10 - 25 Angström) haben.

7. Verfahren nach Anspruch 6, in dem die Donor- und Akzeptorgruppen auf einer Einzelsonde sind.
- 5 8. Verfahren nach Anspruch 6, in dem die Donor- und Akzeptorgruppen auf einem Paar von Sonden sind.
9. Verfahren nach Anspruch 6, 7 oder 8, in dem die Donorgruppe Fluorescein und die Akzeptorgruppe Texasrot ist.
- 10 10. Verfahren nach Anspruch 1 oder 6, in dem die Donor- und Akzeptorgruppen auf einer Einzelsonde und an andere Baseneinheiten als an die 3'- und 5'- Endeinheiten gebunden sind, wobei sie durch 4 - 6 dazwischenliegende Baseneinheiten getrennt sind.
- 15 11. Verfahren nach Anspruch 1 oder 6, in dem die Gruppen auf einem Paar von Sonden mit 3'- bzw. 5'- Enden, die mit benachbarten Baseneinheiten der Zielpolynukleotide hybridisieren, angeordnet sind, und wenn sie so hybridisiert sind, die Donor- und Akzeptorgruppen durch 4 - 6 dazwischenliegende Baseneinheiten getrennt sind.
- 20 12. Verfahren nach Anspruch 10 oder 11, in dem die Donorgruppe Fluorescein und die Akzeptorgruppe Texasrot ist.

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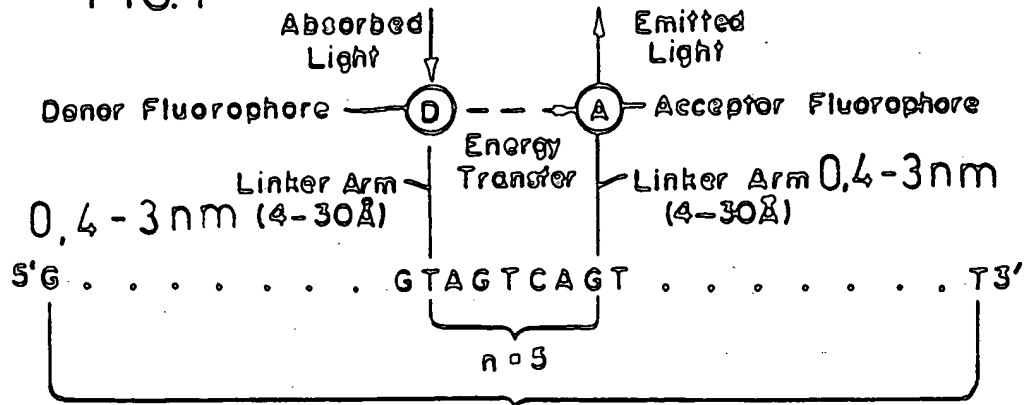
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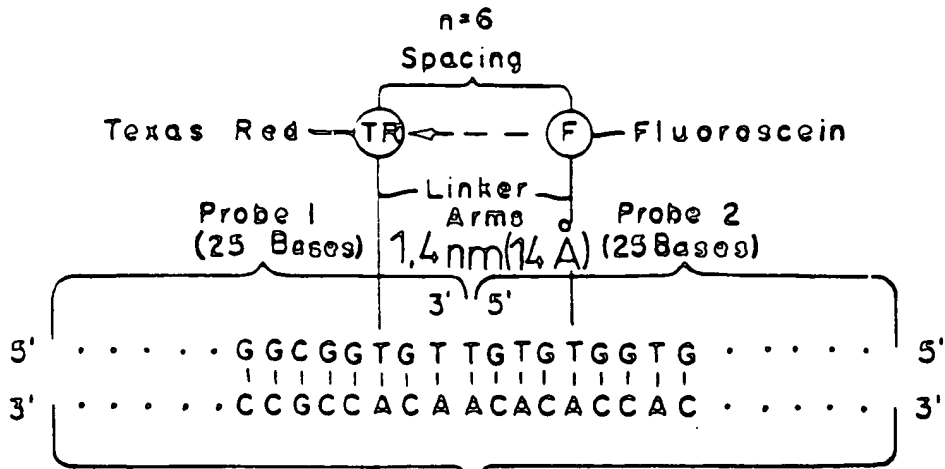
FIG. 1



10-100 Nucleotide Bases

SINGLE PROBE WITH FIVE BASE
(n=5) SPACING OF FLUOROPHORES

FIG. 2



Target Polynucleotide

DUAL PROBES HYBRIDIZED
TO TARGET POLYNUCLEOTIDE SEQUENCES
WITH SIX BASE (n=6) SPACING

FIG. 3

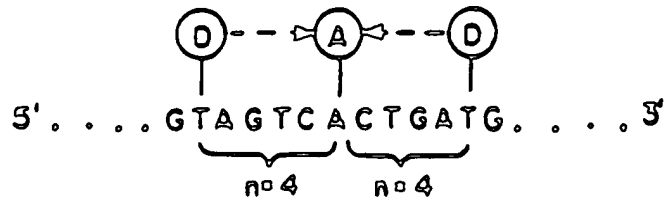


FIG. 4

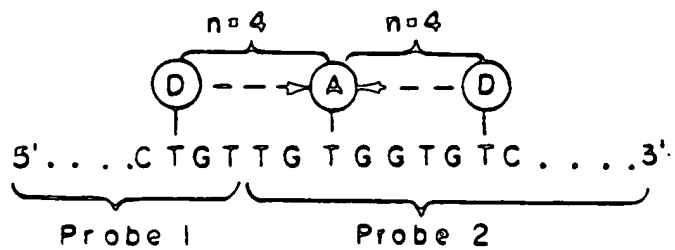


FIG. 5

